

Structural study of Fimbrial Adhesin SafD

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Abstract

Salmonellosis is a major manifestation of enteric disease in humans across the world. *Salmonella* possess multiple fimbriae on their surface and many of which play a vital role in attachment with enterocyte of host tissue to establish and maintain a successful infection. The most common virulence strains of *Salmonella* assemble on their surface. *Salmonella* atypical fimbriae (Saf), consisting of the major SafA and minor SafD subunits. The Saf fimbriae, as many other virulence organelles, are assembled via the chaperone/usher pathway. The SafB periplasmic chaperone assists in subunit folding and assembly, while the SafC usher acts as a fibre assembly platform and a secretion channel. The SafA subunit has been shown to form the main shaft of the fimbriae. The assembly of SafA subunits is based on the principle of donor strand complementation (DSC). The subunit has incomplete immunoglobulin (Ig) fold and hence possess a large hydrophobic “acceptor” cleft. In the fibre, each subunit inserts an N-terminal sequence into the acceptor cleft of the neighbouring subunit hence completing the Ig fold with a donor strand. Although SafD has been shown to act as a highly protective antigen against *Salmonella enteritidis*, the function, structure, and location of SafD in Saf fimbriae are not known. In this study, we expressed SafD fused with the N-terminal donor sequence of SafA (SafD-dsSafA) and analyzed its structure. Our temperature and chemical denaturation experiments demonstrated that SafD-dsc is a stable protein. The successful complementation of SafD with the SafA donor strand strongly suggests SafD is complemented by SafA that in the native fibres. On the other hand, mass spectrometry analysis and modelling revealed that SafD does not contain any own N-terminal sequence that could play role of a donor strand. Hence, in contrast to SafA, SafD cannot form a polymer. Based on these facts, we propose that SafD is situated at the tip of the SafA shaft. Such a special location of SafD suggests that it might play an important role in adhesion or invasion. To check this hypothesis, we aimed at determining a high-resolution structure of SafD, its location on the cell surface of *Salmonella*, and potential SafD receptors on the host cells. We have obtained 2 Å diffracting crystals of SafD and its structure determination is in progress. The SafD-dscSafA construct might be useful to design novel subunit vaccines and rapid diagnostic kits against most common *Salmonella* virulence strains.

Introduction

Salmonella enterica is a member of genus *Salmonella* which is subdivided into seven subspecies and designated as I, II, IIIa, IIIb, IV, VI and VII. Members of *Salmonella enterica* subspecies I are mostly associated with warm blooded animals. Almost 100% of all disease producing salmonella isolates is strains of this subspecies I including serovars *Typhimurium* and *Enteritidis* which are the major causal agents of *Salmonella* induced gastroenteritis in Humans.¹

Pathogenic bacteria use versatile mechanisms to infect their hosts. Surface organelles such as lipopolysaacharide, capsules, surface layers, flagella and fimbriae or pili (Figure 1) play key roles in establishing infection.⁴



Figure 1: *Salmonella enteritidis* with lots of fimbriae.
Source: <http://www.salmonellablog.com/probiotics.jpg> (18.08.2010)

Often, to infect their hosts most pathogenic bacteria need to attach to the target tissues by binding to receptors expressed on the host cell surface. The binding is mediated by special surface molecules or organelles^{2, 3}. Among them, adhesive fimbriae (originated from the Latin word thread or fringe) are the most common. These are proteinaceous, multi-subunit structures that play role in forming contact with host cell receptors (figure 2), hence making bacteria virulent^{1,4,6,7,13} and resistant to antibacterial treatment.⁸

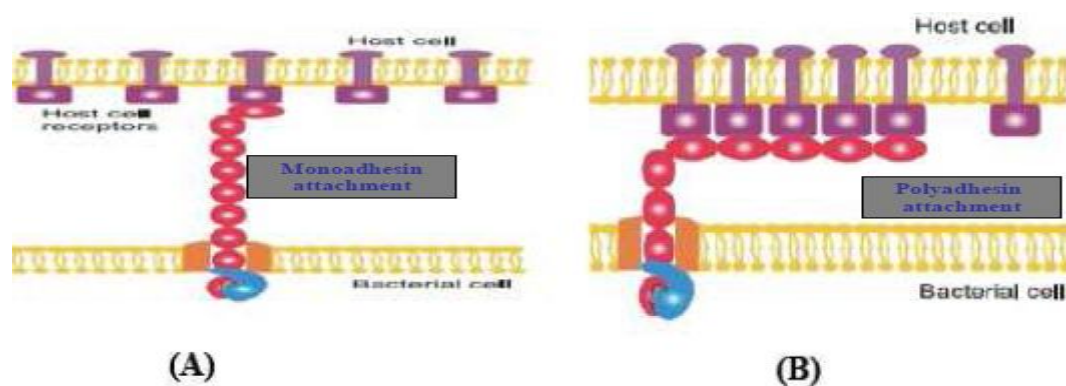
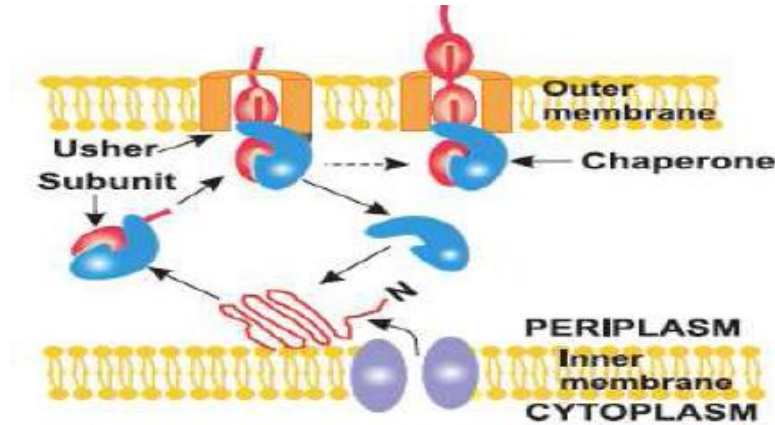


Figure 2: A schematic diagram illustrating the fimbriae mediated attachment of bacteria to host cell receptors. Pathogenic bacteria often bind to epithelial cells e.g. of the gut or bladder. A) Monoadhesin attachment with enterocyte receptor. B) Polyadhesin attachment with enterocyte receptors. Violet colour rectangle represents cell receptor and red coloured oval shape represents the fimbrial subunit(s) of the fimbriae. Redrawn based on Zavialov et al, 2007.

In addition to adhesion and biofilm formation, fimbriae may promote host cell invasion, cell motility, and transport of protein and DNA across the membranes^{6,7}.

Virulence fimbriae can be divided into five major classes according to their biosynthetic pathways and they are chaperone-usher (CU) pili/fimbriae, curli, type IV pili, type III secretion needle and type III secretion pili/fimbriae. The CU pili/fimbriae are the most versatile and the best studied⁷.

The chaperon is a periplasmic protein that facilitates efficient folding of pilus subunits (pilin) resulting stabilization to prevent the aggregation and/or degradation, caps its interactive surfaces to inhibit the premature fibre formation and finally leads to assembly⁸. Usher is a pore forming protein that acts as an assembly platform: it binds the chaperon-subunit complexes, helps in uncapping the chaperon to promote subunit polymerisation, and finally translocates the pilus through the outer membrane (see figure 3)^{7,8}.



Chaperone–usher dependent pathway (Zavialov *et al.* 2007)

Figure 3: Illustration of the mechanism of the fimbriae assembly and secretion via the chaperon –usher pathway. Redrawn based on a figure in Zavialov et al 2007.

The building blocks of fimbriae, the protein subunits, are produced in the cytoplasm and transported to the periplasm through the general secretory pathway¹⁰. Fimbriae subunits possess an incomplete immunoglobulin (Ig) like fold, which is naturally devoid of the 7th C-terminal β -strand (Figure 4). The lack of this C-terminal β -strand produces a large hydrophobic large groove, which makes the subunit highly unstable. To form a complex with the subunit, the periplasmic chaperon inserts a motif of 3-5 alternating hydrophobic residues located on its G1 strand into the groove. This interaction is called donor strand complementation (DSC). Most CU subunits contain 10-20 residues long N-terminal extension (Nte) peptide that is not part of subunit fold. During subunit-subunit polymerization the C-terminal β -strand is replaced by the Nte peptide on the coming chaperon-subunit complex. This mechanism is called donor strand exchange (DSE)^{7, 8, 9, 16}. The details of how chaperon and subunits exchange the C-terminal β -strand and Nte are not known.

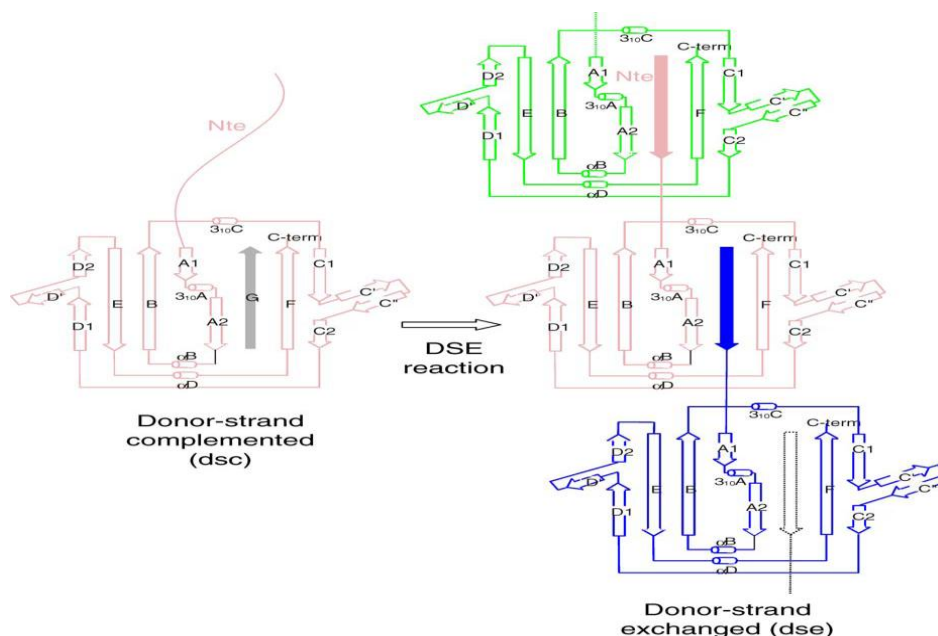


Figure 4: Schematic presentations of donor strand complementation and donor strand exchange. In this topographic diagram arrows represent the β -strands of sheets and cylinders represent helices. The pink topograph on the left represents a subunit complemented by the G donor strand of the chaperone (grey). The G strand of the chaperon runs parallel to the F strand of the subunit. Since this binding creates a complete atypical Ig-like fold, it is called donor strand complementation (DSC). The N-terminal extension of the subunit in the complex is disordered, while in the fiber, it occupies the groove (top right, green and dark pink). The assembly requires exchange of the donor strand of the chaperone to that of the subunit. This process occurs at the usher and is called the donor strand exchange. Redrawn based on a figure in Verger *et al.*, 2007.

Salmonella atypical fimbriae (Saf) are expressed by some strains of *S. enterica* subspecies I via the chaperon-usher (CU) pathway (figure 5)^{9,16}. Saf fibers consist of the major SafA and minor SafD subunits¹. The periplasmic chaperon, SafB and outer membrane protein usher SafC assist in Saf assembly^{1,9}. The SafA subunit has been shown to form the main shaft of the fimbriae¹⁶. The assembly of SafA subunits is based on the principle of DSC. Immunisation of mice with SafB-SafD complex showed significant protection against *Salmonella enteritidis*¹. Neither the function nor the structure of SafD or its location in Saf fimbriae are known yet. SafD has 24 % identical residues with *E. coli* invasins AfaD^{12,16}. Based on the structural information about AfaD and homology modelling Zavialov *et al.* 2007 and Salin *et al.* 2008 suggested that SafD might locate at the tip of the SafA shaft. However, no experimental evidences are available to support this model.

The aim of this study was to determine the structure of the SafD subunit and its location in the Saf fibre. This study will create a solid ground for elucidation of the role of SafD in *Salmonella* pathogenesis, determining the SafD receptors on the host enterocyte. Eventually in the long run, the study may lead to developing new effective vaccine, antimicrobials, and accurate and rapid diagnostics against most common *Salmonella* virulent strains.

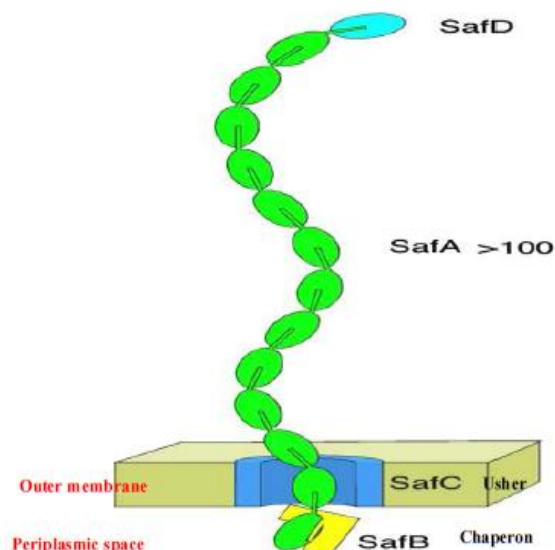


Figure 5: The schematic representation of chaperon-usher assembled Saf pilus proposed by Salih *et al.*, 2008. Subunits are represented as green oval shape form. The yellow rectangular protrusion is the N-terminal extension (Nte) of subunit. The SafB chaperon-subunit complex is targeted to the usher assembly platform at the outer membrane. Subunits polymerise by donating Nte to the next subunit and secrete through the channel in the SafC usher. The major subunit SafA is shown in green, the chaperon SafB in yellow, usher SafC is in grey colour. SafA (maximum in number) and SafD made the outer shaft of the pilus where SafD located at the very distal end of the pilus. Redrawn based on a figure in Salih *et al.*, 2008

Materials and Methods

1. Isolation and purification of SafD-dsc gene fragment

SafD-dsc gene fragment was isolated by PCR method. pfuUltra DNA polymerase had been used as enzyme, SafD F1 forward and SafD reverse were used as primers. The sequences of the primer are given below in table 1. The reaction mix for PCR and PCR program that had been used are given below in table 2 and table 3 respectively.

Table.1 list of primers with sequences

<u>Primer</u>	<u>Sequences</u>
SafD (2)forward	5'-CACCATGAAAACGGTTATCTATAGCGTAAC C'-3
SafD reverse	5'-CTAAGCGGCAAAGCTAACATTAC-3'

Table.2 PCR reaction mix ingredients

<u>Ingredients</u>	<u>Amount in µl</u>
Sterile water	40
10x Pfu buffere	5
dNTPs (10mM)	1
SafD(2) forward	1
SafD reverse	1
Template DNA	1
Pfu ultra DNA polymerase	1
Total volume	50 µl

Table.3 PCR programme

<u>Steps</u>	<u>Cycles</u>	<u>Temperature and time</u>
Initial Denaturation	1	94°C for 2 min
Denaturation		94°C for 1 min
Annealing	30	50°C for 1 min
Elongation		72°C for 1 min
Final elongation	1	72°C for 10 min
Hold in		4°C

2. Agarose gel electrophoresis

The amplified PCR product was analysed by 1% agarose gel. Agarose gel was prepared as described here; 1 gram ultrapure agarose powder (Invitrogen, USA) was taken in a 250 ml volume conical flask and boiled with 100ml 1xTAE buffer until agarose melted. The flask was cooled down to convenient to touch by hand then 40 ml was taken in a beaker and 4 µl of GelRed (Biotium, USA) was added and mixed properly. After that the agarose was poured down in a gel casting tray. 1xTAE buffer was used as gel running buffer. Total sample volume was 60 µl (50 µl PCR product and 10 µl 6x loading dye). Samples were loaded and 100bp plus DNA marker was loaded in the first well. Electrophoresis was run for 45 min with a current of 100 V. After that gel was analyzed under UV-transilluminator.

3. Purification of DNA fragment from agarose gel

Eppendorf tube was weighed and marked on the body, expected DNA band was cut down by clean and sharp scalpel and put in eppendorf tube, weighed again the tube to know the actual weight of DNA band containing gel piece. DNA fragment was purified by using standard QIAquick Gel Extraction Kit (Qiagen, USA). Gel piece was dissolved with 3 volumes QG buffer by incubated in 50°C for 15 min followed by transferred to QIAquick spin column to bind the DNA fragment. The spin column had been centrifuged by 13000 rpm (by table top centrifuge machine) for 1 min. After that the column was washed up by 0.75 ml of PE buffer and centrifuged for 1 min, the flowthrough was discarded and centrifuged again for 1 min. The spin column had been transferred to a clean eppendorf tube and 50 µl sterile water had been added in the middle of the column, after 1 min incubation in room temperature, the eppendorf tube with spin column had been centrifuged at 13000 rpm for 1 min. Concentration of the DNA fragment had been measured by spectrophotometer (Nanodrop 1000).

4. Cloning of SafD-dsc in to pET101/TOPO vector

TOPO cloning ligation reaction mixture had been prepared as below.

Table.4 list of ingredients for ligation reaction

<u>Name of ingredients</u>	<u>Amount (µl)</u>
PCR product (3.8ng/ul)	1
Salt solution (Invitrogen, USA)	1
TOPO vector (Invitrogen, USA)	1
Sterile water (Invitrogen, USA)	3
Total	6 µl

PCR product was diluted to the recommended concentration for TOPO cloning reaction. All the ingredients as stated in table 4 were taken in an eppendorf tube, mixed them gently and properly. Ligation mixture was kept in room temperature to incubate for 20 min. Meanwhile TOP10 commercial competent cell was taken and thawed on ice for 10 min. 3 µl of ligation mixture had been added to the TOP10 cell and incubated on ice for 30 min. To increase the porosity of the cell, heat shock was applied for 30 s at 42 °C in water bath without any agitation. After 30 s, the cell was put back into ice for 5 min incubation. 600 µl fresh LB medium was added to the cell and put in a shaker at 37 °C and 200rpm for 1 h. TOPO cells were spreaded on pre-warmed LB-agar plate (ampicillin 100mg/ml included) and kept until dried. Plate was incubated at 37 °C for overnight.

5. Positive transformation screening through Colony PCR

Colony PCR method was used to screen the positive transformation. One bacterial colony was fished out and put into a sterile glass tube containing 3 ml LB medium and 3 µl ampicillin (100 mg/ml). Incubated at 37 °C and 200 rpm for overnight. On the following day, 10 µl culture was taken and boiled at 95 °C for 5 min to use as template DNA in PCR reaction. After boiling the sample was kept on ice. In general T7 forward and SafD reverse primers were used but to confirm minimum amplification, V5 reverse and T7 forward primer were used as control. The sequences of primers in table 5a, ingredients with amount and PCR program were given in table 5b and table 6 respectively.

Table5a: Sequence of primers

<u>Primer</u>	<u>Sequence</u>
T7 forward	5'-TAATACGACTCACTATAGGG-3'
SafD reverse	5'-CTAAGCGGCAAAGCTAACATTTCAC-3'
V5 reverse	5'-ACCGAGGAGAGGGTTAGGGAT-3'

Table5b List of ingredients used in Colony PCR

<u>Ingredients</u>	<u>Amount (μl)</u>
Sterile water	19.8
10x standard Taq buffer	2.5
T7 forward primer (20pmol)	0.5
SafD reverse primer (20pmol)	0.5
dNTP(16mM)	0.5
Template DNA	1.0
Taq DNA polymerase	0.2
Total	25 μl

Reaction mixture for V5 reverse primer, V5 (20 pmol) reverse primer was used instead of SafD reverse primer and rest of the ingredients and the amounts were same as stated in the above table.

Table 6. Programme for colony PCR.

<u>Steps</u>	<u>Cycles</u>	<u>Temperature and Time</u>
Initial Denaturation	1	94°C 2 min
Denaturation		94°C 1 min
Annealing	30	50°C 1 min
Elongation		72°C 1 min
Final elongation	1	72°C 10 min
Hold in		4°C

6. Plasmid purification

600 μl culture had been taken for making glycerol stock. Plasmid was extracted from the competent cells by using QIAminiPrep Kit (QIAGEN, USA). 3 ml overnight culture was taken in a 15 ml sterile Falcon tube followed by centrifugation at 4500 rpm for 10min. Supernatant was discarded and cell pellet was resuspended by adding 250 μl P1 buffer and transferred to a microcentrifuge tube. After that 250 μl P2 buffer was added and mixed by inverting the tube 4-6 times. Buffer N3 (350 μl) was added and mixed properly by inverting the tube 4-6 times followed by centrifugation at 13000 rpm for 10 min. Carefully transferred the supernatant from the micro centrifuge tube to the spin column and centrifuged at 13000 rpm for 1 min. Flowthrough was discarded. The spin column was washed by adding 750 μl PE buffer and centrifuged at 13000 rpm for 1 min. Additional 1 min centrifugation had been done to remove the extra PE buffer residue. For elution 50 μl of sterile water was added at the middle of the spin column membrane. Following 1 min incubation, the

spin column was centrifuged at 13000 rpm for 1 min. The flow through was the plasmid. Concentration was measured by spectrophotometer (Naodrop1000). Eppendorf tube was named as pETSafD-dsc with concentration amount. Plasmid had been stored in -20°C.

7. Preparation of glycerol stock

After colony PCR and agarose gel run, it was clear that which colony gave positive result. Glycerol stock had been prepared from those positive clones. 200 µl of sterile 88 % glycerol was taken in an eppendorf tube and 600 µl of overnight culture was added. Vortexed properly and flash frozen with liquid nitrogen for a while before stored in -80 °C.

8. Transfer plasmid into BL21AI expression cell

Purified plasmid was transferred into BL21AI cell by heat shock method. BL21AI home made cell had been taken from -80°C and put on ice for a while to thaw, after thawing, 1 µl (33ng/µl) of plasmid was added to the cells and mixed by tapping. Put the cell on ice for 30 min incubation. After that the cell was incubated in water bath at 42°C for exactly 30 s without any agitation. After 30 s the cell was put back on ice immediately. 400 µl fresh LB medium was added with the cell and placed on a shaker at 37 °C and 220 rpm for 1 h. After 1 h, cell culture was spreaded over a pre-warmed agar plate and kept inverted at 37 °C for over night incubation.

9. Test Expression in BL21AI cell

Three small (15ml) falcon tubes were taken and filled with 4 ml fresh LB medium plus 4 µl ampicillin (100 mg/ml). Two tubes were for SafD-dsc and 3rd one was for SefD-SC as control. One colony was taken from each respective plate of SafD-dsc(s) and SefD-SC and mixed with LB medium in each falcon tube. Finally put in a shaking incubator for over night at 37 °C and 200 rpm.

Six 100 ml volume sterile glass flasks were taken and filled with 23 ml of fresh LB medium plus 23 µl of ampicillin (100mg/ml), after that 1 ml of overnight culture was added in each flask as four for SafD-dsc and other two for SefD-SC as control. Put all the flasks in a shaking incubator, for smooth growing of bacterial cells, at 37°C and 150-200 rpm (rpm may vary depending on incubator size, big incubator low rpm and small incubator high rpm). After 1 h the Optical Density (OD) of culture was measured (OD around 0.55), put back cultures and temperature was reduced to 28°C, cells were allowed to grow in this temperature for 30 min before induction. After this 30 min the OD was around 0.9 at wavelength 600 nm. One culture from each was induced with 1M IPTG 21 µl (to make final concentration 1 mM) and 20% arabinose 200 µl (to make final concentration 0.2%). And other three cultures were kept uninduced as negative control. The cultures were kept at 28°C and 120-160 rpm for 4 h for expression of protein.

10. Protein harvesting from periplasmic space

After 4 h expression, 10ml culture was transferred from each flask to new sterile 15 ml falcon tube with proper naming followed by centrifugation at 4500 rpm for 15min. The supernatant from each tube had been discarded and the cell pellet in each tube

was resuspended with 600 μ l of 20 % (w/v) sucrose + 50 mM Tris-HCl pH 8.0 + 5 mM EDTA solution. After proper mixing with sucrose solution, the resuspended cell pellet from each tube was transferred to new eppendorf tubes with proper marking and kept on ice for 10 min incubation. After 10 min all the tubes were centrifuged at 5000 rpm for 15 min, supernatant was collected as sucrose fraction with proper marking for SDS-PAGE analysis. The cell pellet was resuspended (for each tube) with 400 μ l 5 mM $MgSO_4$ and incubated on ice for 10 min followed by 5 min centrifugation at 10000 rpm. The clear supernatant was collected as periplasmic fraction in new eppendorf tubes.

11. SDS-PAGE (15%) to analyze the expression of protein

Table 7: List of ingredients to prepare 15% gel

15% separating gel pH8.8	Amount in ml	4% stacking gel pH6.8	Amount in ml
88% glycerol	0.525	Water	1.84
1M Tris-HCl, pH 8.8	1.87	1M Tris-HCl, pH 6.8	0.312
30% AA-bisAA(29:1)	2.5	30% AA-bisAA(29:1)	0.325
10% SDS	0.05	10% SDS	0.025
10% PSA	0.05	10% PSA	0.025
TEMED	0.005	TEMED	0.002
Total	5 ml	Total	2.5 ml

Sample was prepared for SDS-PAGE gel as 30 μ l sample, 10 μ l 4x loading dye and 1 μ l 2-mercapta-ethanol in one eppendorf tube followed by 5 min boiling at 95 °C. After boiling 10 μ l from each sample was loaded in the gel well. Low molecular weight marker was loaded in the 1st lane. After that the electrophoresis was run at 200 V for 1 h in 1x SDS Running buffer (25 mM Tris-base + 192 mM Glycine + 0.1% SDS + H_2O)

12. Staining and destaining of the gel

The gel was removed from the gel device carefully and put in an oven proof plastic box; some fixing solution (30 % ethanol and 10 % acetic acid) was poured down upon the gel. The gel was heated until boiling, put back the fixing solution and some Coomassie blue solution was added and heated until boiling, after that the gel was put on a shaker with Coomassie blue solution for 5 min. After 5 min, the Coomassie blue solution was restored and the gel was rinsed with water followed by heated with water until boiling. Finally the gel was kept on shaker for 5 min or longer to visualize the band clearly.

13. Over expression and purification of protein

For large culture, overnight culture was set on the previous night in a 500 ml flask with 200 ml fresh LB, 200 μ l ampicillin (100 mg/ml) and a few respective cells (BL21-AI/pETSafD-dsc) from glycerol stock. Put the flask in a shaking incubator at

37 °C and 220 rpm. On the following morning, 1 l dry Oven sterilized (250 °C and 12 h) flask was taken and filled with 900 ml fresh LB medium along with 900 µl ampicillin (100mg/ml). 30 ml of overnight culture was added with LB medium and put for growing cells in a big shaking incubator at 37 °C and 140 rpm. Optical density of the culture was checked by UV spectrophotometer after 90 min and it was around 0.6 at 600 nm wavelength. The flask was transferred to another shaking incubator at 28°C and 120rpm for 30 min or until the OD reached at around 0.9 at 600 nm wavelength. The culture was induced with 920 µl 1M IPTG (1mM final concentration) and 9.2 ml of 20 % arabinose (0.2% final concentration). The cells in the culture were allowed to express protein up to 4 h at 28 °C and 120 rpm. After 4 h the culture was taken in a 1 l plastic bottle and centrifuged at 4500 rpm for 30min. The supernatant was discarded and the cell pellet was resuspended with 20 ml of 20 % (w/v) sucrose + 50 mM Tris-HCl pH 8.0 + 5 mM EDTA solution. After re-suspension the cell was incubated on ice for 10 min and centrifuged at 6800 rpm for 20min. After centrifugation the supernatant was collected in one bottle as sucrose fraction for SDS-PAGE analysis. The cell pellet was then resuspended with pre-cooled 5 mM MgSO₄ (for Osmotic shock) and Protease inhibitor tablet solution. Incubated on ice for 10 min and had been centrifuged at 7500 rpm for 20 min. The clear supernatant was collected in a bottle as periplasmic protein extraction.

14. Dialysis and filtration of protein

Protein sample was dialysed over night in a molecularporous dialysis membrane (SPECTRA/POR) to reduce the salt concentration and adopted with the loading buffer pH for next chromatography. After each dialysis, protein sample was filtered with 0.45 µm and 0.2 µm filter respectively.

Table8: list of buffer that was used for dialysis in different steps of protein purification.

<u>Chromatography</u>	<u>Name of Buffer for dialysis</u>
Ion (anion) exchange	20 mM Tris-HCl pH 8.0
Ion (cation) exchange	40 mM Sodium acetate pH 4.5

15. Protein purification by liquid Chromatography

Automated AKTA Explorer system was used to perform chromatography.

Anion-exchange chromatography

Source 30Q, 20 ml column was used in the first purification step. The column was washed with 3 column volume (CV) elution buffer (20 mM Tris-HCl Ph 8.0 and 1 M sodium chloride) and followed by equilibration with 3 CV loading buffer (20 mM Tris-HCl pH 8.0). Dialysed and filtrated periplasmic protein sample was loaded into the column at the flow rate of 1 ml/min. The sample was loaded and then the column washed with 1.5 CV of loading buffer to carry the protein from the loading tube in to the column. The protein was eluted at the flow rate of 1 ml/min and a gradient of 30 % for 100 min. After purification, SDS-PAGE was run to identify the correct peak and fractions of eluted protein. Put the protein sample in the dialysis membrane with appropriate buffer for next chromatography step.

Cation-exchange chromatography

A MonoS 1ml column was used. The column was washed with 30 CV elution buffer (40 mM Sodium acetate pH 4.5 +1 M sodium chloride) and followed by equilibration

with 30 CV loading buffer (40 mM Sodium acetate pH 4.5) at the flow rate of 1 ml/min. Dialysed and filtrated protein sample was loaded on to the column at the flow rate of 1 ml/min. After sample loading, again 20 CV of loading buffer was loaded to carry the protein from the loading tube in to the column. The protein was eluted at the flow rate of 0.5 ml/min, fractionation rate 0.5 ml/tube and gradient was set 30% for 30min before elution. After purification, SDS-PAGE was run to identify the correct peak and fractions of eluted protein. Next was polishing step, protein sample was not needed to be dialysed but protein sample was concentrated by using Vivaspinn-6 column 5000 MWCO PES (GE Healthcare).

Size exclusion (Gel filtration) chromatography

In this chromatography, Superdex 75E 120 ml column (GE Health Care) was used to polish the protein. The column was equilibrated with 150 ml of buffer 50 mM Sodium acetate pH 4.5+ 150 mM Sodium Chloride at the flow rate of 1.5 ml/min. 2 ml concentrated sample was injected into the column and 30 ml buffer had been run to the column before eluting protein fractions. After that polished and pure protein was eluted at the rate of 2 ml/tube (flow rate 1 ml/min). SDS-PAGE was run to analyze the purity of eluted protein.

16. Stability test through Circular Dichroism (CD) spectroscopy

Circular Dichroism is a method that is used to determine the differential absorbance of the components that circularly polarise in L and R orientation. The protein absorbance occurs through the amino acid side chain. Absorbance in peptide bond occurs below 240 nm and absorbance in disulphide bond at 260 nm and absorption at aromatic amino acids at 260 to 320 nm. Secondary structure composition of protein such as helices and different sheets can be identified through CD spectrum. By accumulating all these information, the stability of a protein can be determined. It can be determined by using temperature and chemical denaturant (guanidium hydrochloride, urea etc)¹⁵. For the study of Guanidium hydrochloride (GdnHCl), samples were prepared as shown in the table below.

Table 9. Reaction mix of GdnHCl and protein for CD

<u>Sample</u>	<u>S1</u>	<u>S2</u>	<u>S3</u>	<u>S4</u>	<u>S5</u>	<u>S6</u>	<u>S7</u>	<u>S8</u>
GdnHCl in sample in M	0	0.5	1	1.5	2	2.5	3	3.5
Protein in(0.5 mg/ml) μ l	50	50	50	50	50	50	50	50
20mMPB pH 7.0 in μ l	50	50	50	50	50	50	50	50
8M GdnHCl in μ l	0	12.5	25	37.5	50	62.5	75	87.5
ddH ₂ O in μ l	100	87.5	75	62.5	50	37.5	25	12.5
Total (in μl)	200	200	200	200	200	200	200	200

17. Mass spectrometry had been done to analyse of its molecular weight, signal peptide cut point and N-terminal extension presence.

18. Crystallization

Crystal screening by sitting drop vapour diffusion method:

To find out the better crystal growing condition, a crystal screening was done by Hartman index crystal screening I and II in 96-well plate. 80 μ l of each screening condition mother liquor was taken in each well, after that 1 μ l mother liquor of one well put in the sitting place and 1 μ l of protein (stock 20 mg/ml) was mixed by 2 times pipetting, did the same for the rest wells. This drop possessed only half the amount of mother liquor content. Finally, the wells were sealed to be protected from evaporation with an adhesive transparent sealer and store in room temperature (20°C) without any agitation. While equilibrium sets the water from the drop evaporated to the reservoir and the drop would finally have the same amount of mother liquor.

Crystal growing by hanging drop diffusion method:

After detecting the best condition from the screens, 5 different conditions were prepared by using 5 different percentage of precipitant (PEG 4000) along with salt (0.1 M ammonium acetate) and buffer (0.1 M sodium citrate pH 5.1)(see table 10 for more details). At the beginning, 600 μ l of 5 different mother liquors were taken in 5 wells, each well for each type of mother liquor, after that a glass lid was taken, on the glass lid 2 μ l of mother liquor and 2 μ l of protein were mixed by gentle pipetting, the glass lid was inverted upon the reservoir-well which sealed with grease to make it a airtight condition, after placing the lid on the well, the lid was pressed and move 45° to make sure the air tightness. The setups were incubated at 20°C.

Table 10. List of chemicals for big drop crystal growing. (Optimized from Hartman research screening condition A9)

Chemical	28 % PEG 4000	29 % PEG 4000	30 % PEG 4000	31 % PEG 4000	32 % PEG 4000
1M Sodium citrate pH5.1	100 μ l	100 μ l	100 μ l	100 μ l	100 μ l
1M Ammonium acetate	200 μ l	200 μ l	200 μ l	200 μ l	200 μ l
50% PEG4000	560 μ l	580 μ l	600 μ l	620 μ l	640 μ l
Sterile Water	140 μ l	120 μ l	100 μ l	80 μ l	60 μ l
Total volume	1 ml	1 ml	1 ml	1ml	1 ml

The initial screening condition (A9 in screening kit) pH was 6.4 and after preparing five different conditions with different percentages of precipitant, the pH was 6.0.

Additive screening by sitting drop vapour diffusion method:

After obtaining the best condition with one of PEG 4000 percentages, a 96 well additive screen was done with the Hampton research additive screen (HR2-428 page 1 and 2), according to the protocol with a difference in sample size. In this study, 2 μ l of protein, 1.6 μ l of mother liquor and 0.4 μ l of respective additive were used to set crystal drop.

19. X-ray diffraction of crystal

Crystal was diffracted with home lab facility, BMC, Uppsala, Sweden.

Result

1) SafD-dsc cloning and expression

The SafD-dsc gene fragment was isolated from the available construct pUC57/SafD-dsc in the lab. After isolation of gene fragment and ligation with pET101/D TOPO vector, the vector was transferred to Top10 *E. coli* competent cells. PCR and Colony PCR were done to confirm the fragment isolation and positive vector transformation in to competent cells respectively. PCR products were analyzed on a 1% agarose gel. In figure 6 (left) lane 1 ladder, lane 3 and 5 bands depicted the positive isolation of SafD-dsc gene fragment. Figure 6 (right) upper part lane 1 ladder, lane 2 and lane 4 were band of positive transformant but lane 8 and lane 10 were positive with some contamination. Bottom part, PCR with V5 reverse primer to confirm the amplification, here lane1 ladder, lane 2 and lane 4 were the positive transformant as upper part and lane 8 and lane 10 were positive too with some contamination.

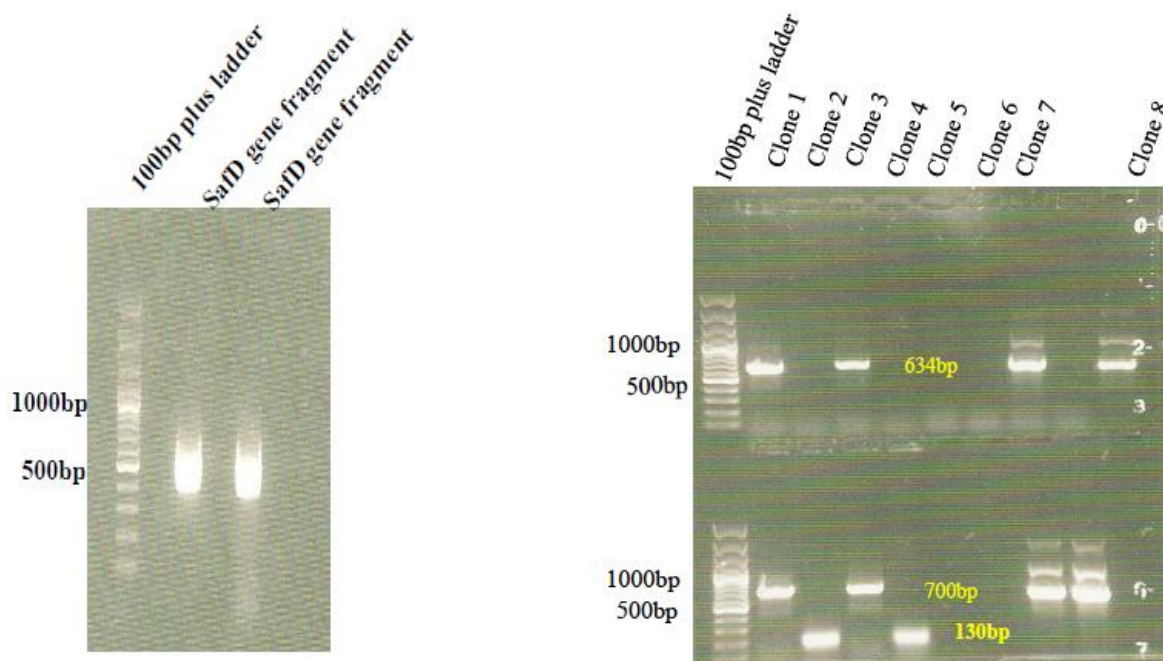


Figure 6: PCR result of SafD-SC gene isolation (left) and Colony PCR result (right) of plasmid pETSafD-dsc.

SafD-dsc test expression:

Test expression of SafD-dsc was performed in BL21AI cells. Different expression temperature was tested but 28°C was found optimal. At the time of test expression SefD-dsc was used as positive control. In the figure 7, lane 1 is low molecular weight marker, lane 3 and 4 are uninduced and induced SefD-dsc periplasmic fraction respectively, lane 5 and 7 are uninduced SafD-dsc periplasmic fraction. Lane 6 and 8 are induced SafD-dsc periplasmic fraction. Uninduced sample was used as negative control.

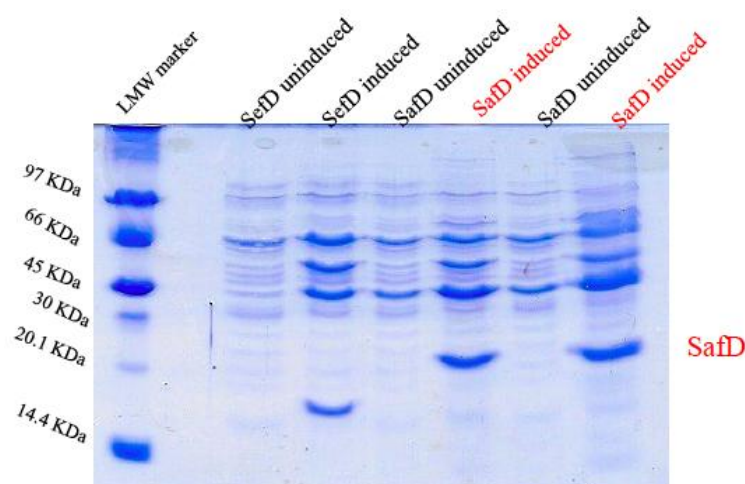


Figure 7: SDS-PAGE result of Test expression of SafD-dsc subunit in periplasmic space.

SafD-dsc overexpression and purification

When harvesting of protein had been done, both the fractions (sucrose and periplasmic) had been analyzed on 15 % SDS-PAGE. In figure 8, lane 1 low molecular weight marker, lane 2, 4, 6, 8 are periplasmic fractions, lane 3, 5, 7, 9 are sucrose fractions. Periplasmic fraction gave thick band compare to sucrose fraction.

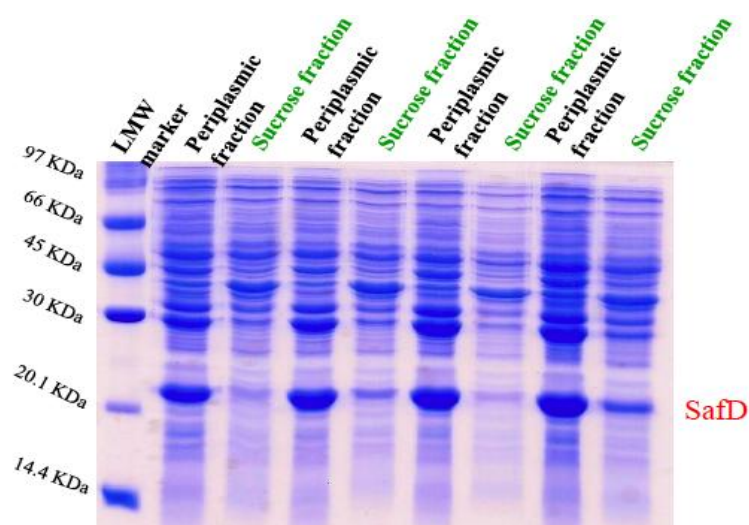


Figure 8: SDS-PAGE result of SafD-dsc expression in periplasmic space.

The sample was then dialysed by Source 30Q loading buffer for over night. After purification by Source 30Q, sample from different peaks had been analysed on a 15% SDS-PAGE and found that lane 6 (figure 9a) contain the maximum protein that represented 4th peak (in figure 9b). Fraction tube 26th from 4th peak is in lane 6, so fractions from tube 25th to 32nd had been polled together.

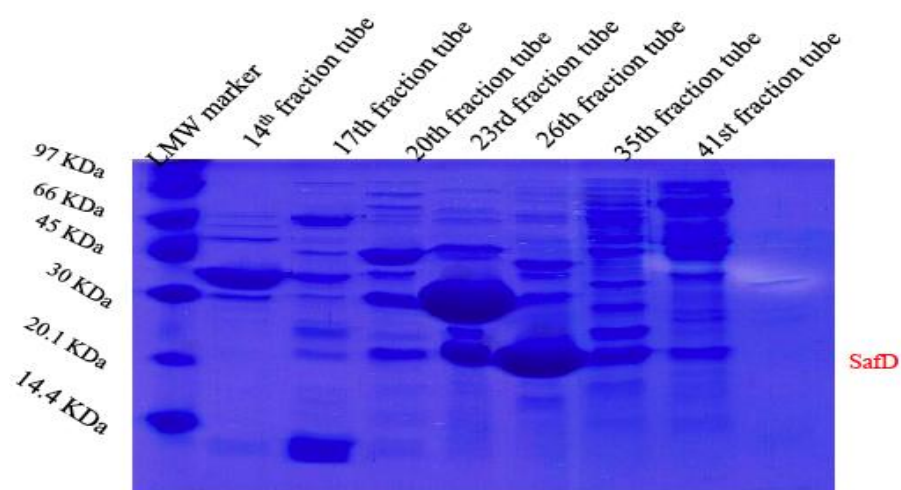


Figure: 9a SDS-PAGE result after Anion exchange (Source 30Q) chromatography.

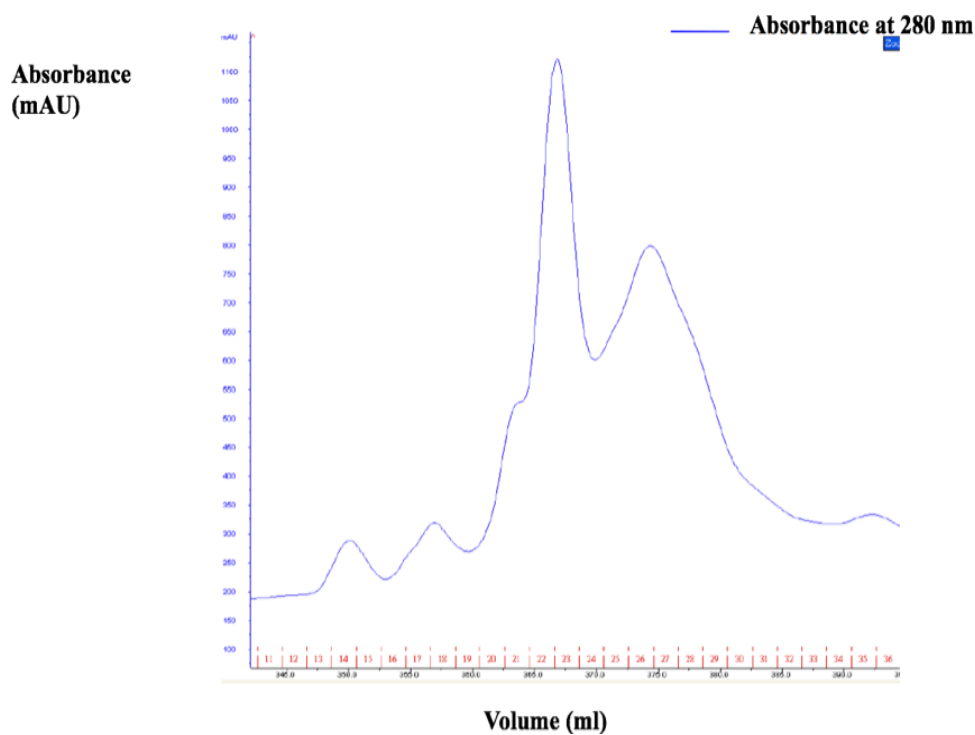


Figure9b: Result of Anion exchange (Source 30Q) chromatography.

After that the sample was dialysed overnight with Mono-S loading buffer. This sample was free of positive charged un-specific proteins and peptide fragments. Only one big peak had been found after cation exchange chromatography with other very small peaks. More than one fraction had been analysed on 15% SDS-PAGE (figure 10a).

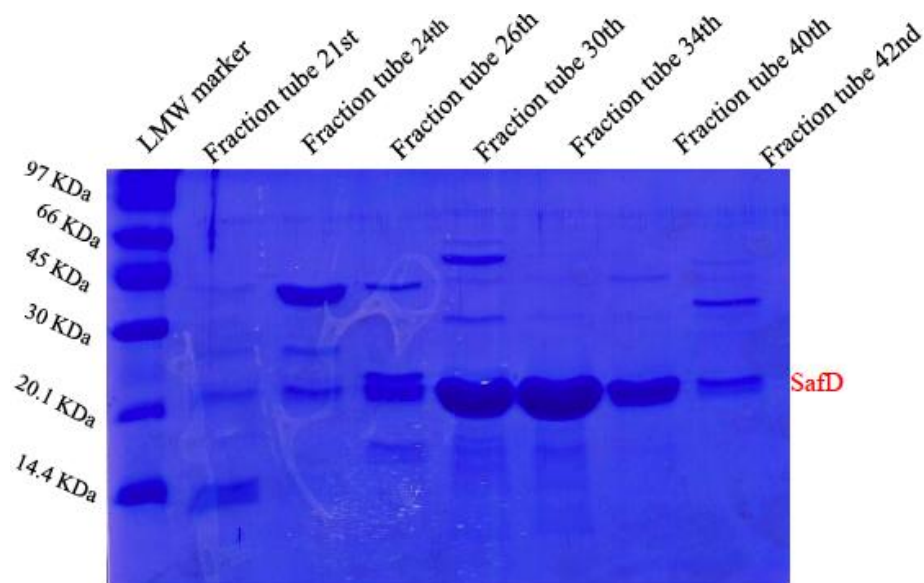


Figure 10a: SDS-PAGE after Cation exchange chromatography

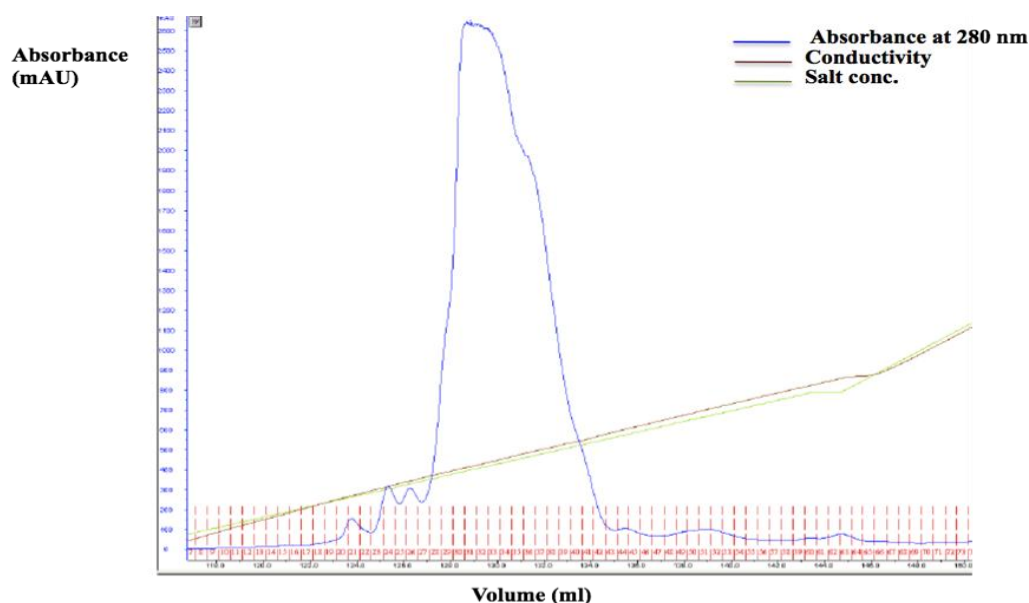


Figure10b: Result of Cation exchange chromatography.

Fractions concerning big peak were 29th to 42nd tube, 0.5 ml size, were pooled together (figure 10b). This sample had been concentrated to 2 ml for Size exclusion chromatography. Size exclusion chromatography separates protein by their size, protein molecule that larger in size being separated and eluted first than the smaller size molecules. After this chromatography, protein was eluted as 2 ml fraction size and gave a large peak from fraction tube 18th to 24th (figure 11b). To avoid any kind of contamination, fractions 19th to 22nd had been taken as purest protein. This purity was analyzed on a 15% SDS-PAGE. After gel electrophoresis, it gave only one band (figure 11a)

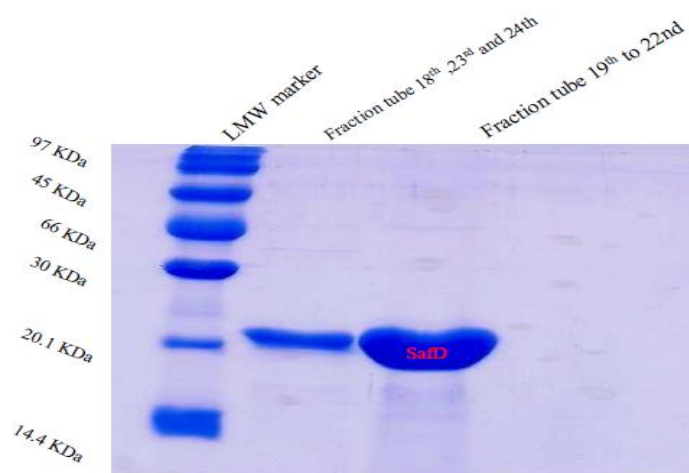


Figure 11a. SDS-PAGE result after Size exclusion chromatography.

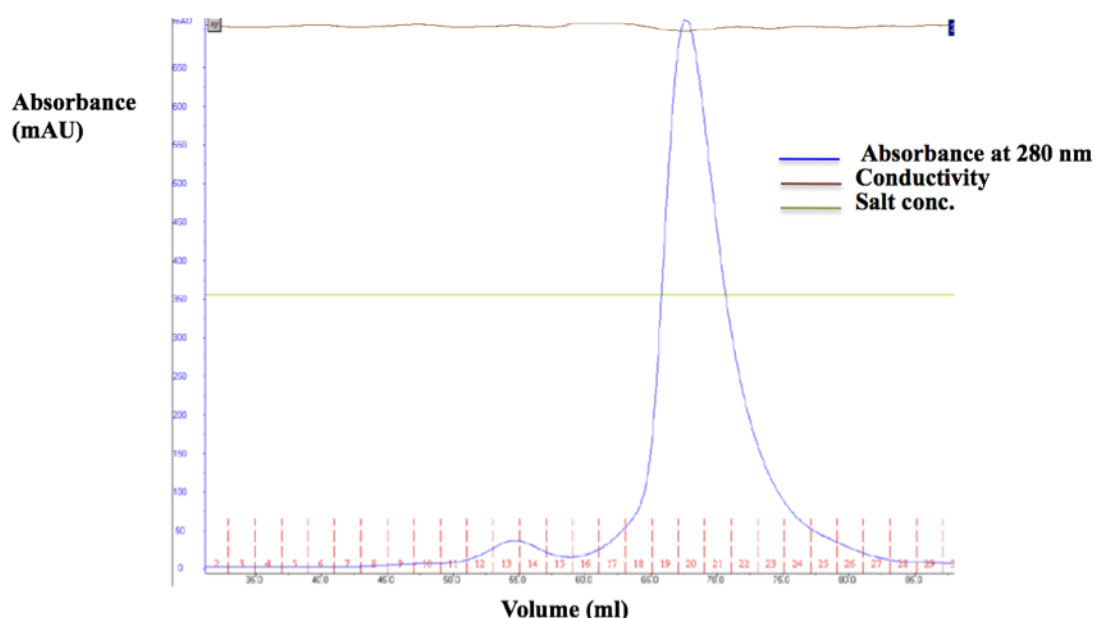


Figure 11b: Result of Size exclusion chromatography.

The pooled sample was concentrated to make 20 mg/ml to volume 350ul. Aliquoted them as 50 μ l in eppendrof tube with proper naming, after flash freezing in liquid nitrogen, stored in -20°C .

Stability test

Stability test of SafD-dsc was done by temperature and chemical denaturants, guanidium hydrochloride. For temperature denaturation, diluted protein was used in the CD, after incubation of the cell in the chamber, the temperature had been increasing gradually and at a certain stage the CD signal became steady. From the figure 12a it was calculated that SafD-dsc protein remains stable to 55°C and above that temperature it began unfolding. In case of Guanidium hydrochloride, it was found

that SafD-dsc protein began denaturing at 2 M Guanidium hydrochloride concentration (figure 12b). SefD was used to compare with and as a positive control.

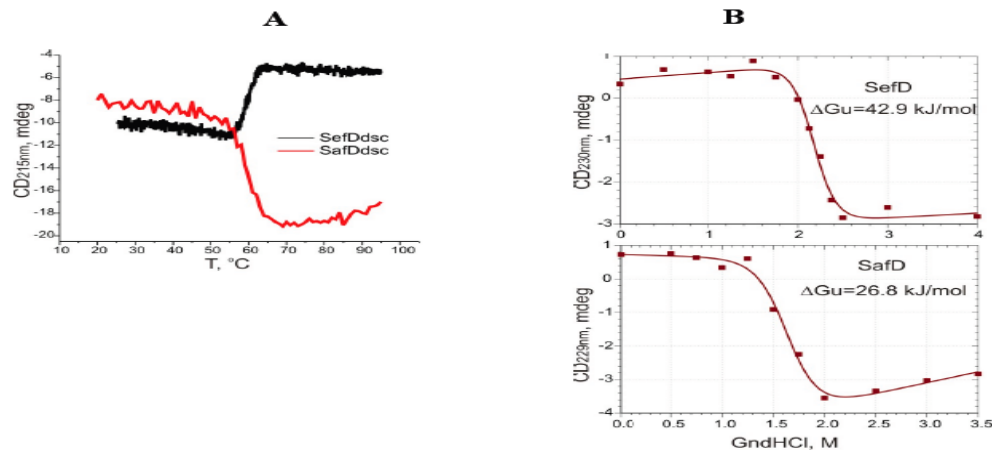


Figure 12: Stability test result by using CD spectroscopy. A) With temperature denaturant; B) with chemical denaturant.

Mass spectrometry:

Mass spectrometry analysis revealed that the molecular weight of this protein is 16.24 KDa (Figure 13a).

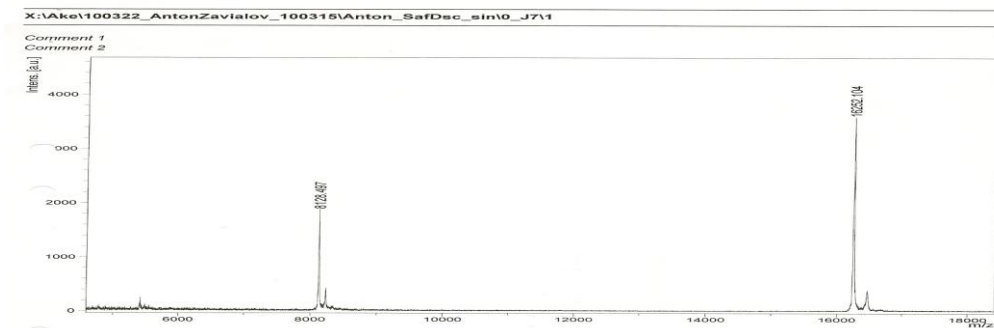


Figure 13a: Mass spectrometry results.

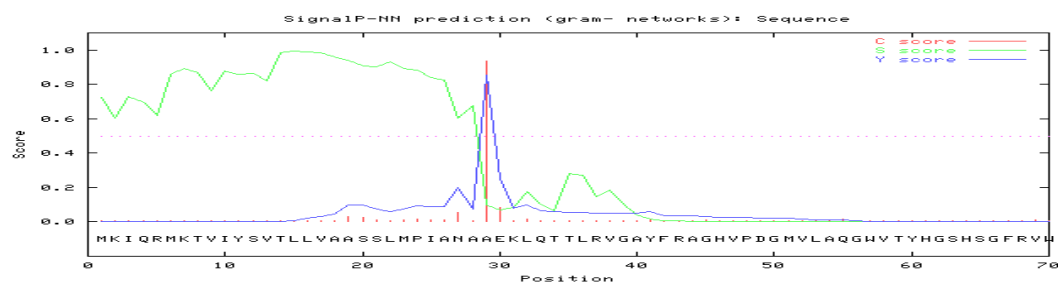


Figure 13b: Signal peptide cleavage site of SafD-dsc, cleaved between ANA and AE.

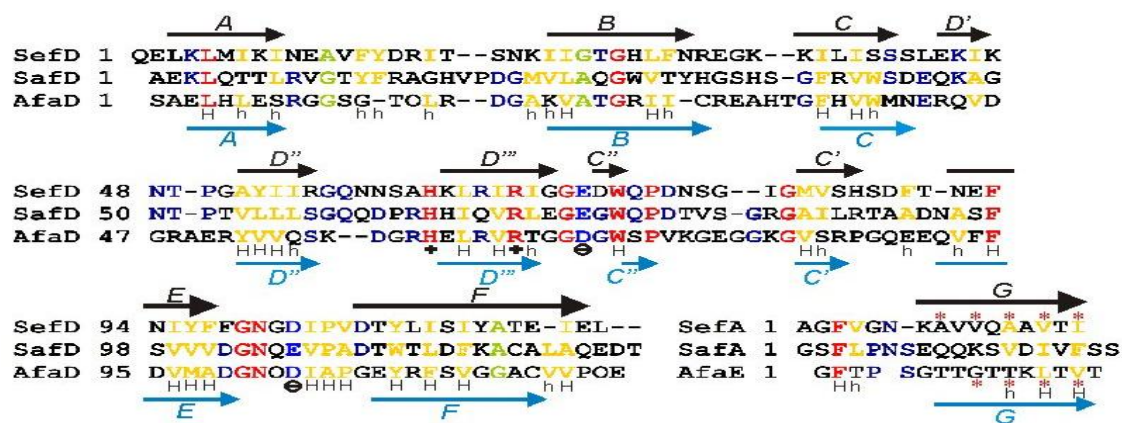


Figure 14: Multiple alignment with SefD and AfaD.

The protein is processed between Ala28 and Ala29 position (Figure 13b). SafD does not have any N-terminal extension sequence. Multiple sequence alignment with SefD and AfaD (figure 14) strongly suggested that SafD could not have any N-terminal extension. SefD and AfaD structure have been solved.

Crystallization and diffraction

Lots of crystal were obtained from condition A9 (Hampton research) (PEG4000, 28% to 32%) and found that PEG4000 28% and 29% were least crowded. So PEG4000 28.5% was taken for 96 well additive screening, after additive screening (Hampton research) , it was found that almost 40 additive conditions produced crystal (figure16) but their morphology were as same as without additives. A small crystal from A9 condition (PEG4000, 29%) (figure 15) was diffracted with home lab facility and the image (figure 17) was so far promising with resolution 2 Å.

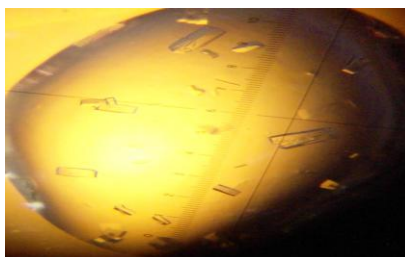


Figure 15: Crystal from A9 condition



Figure 16: Additive screened crystal (left A7, middle E10, right G10 condition)

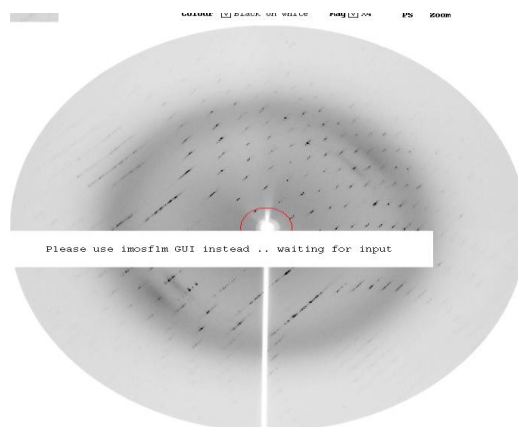


Figure 17: Diffracted image of crystal

The diffracted image (figure 17) is quite promising to solve the structure. The structure determination is in progress.

Conclusions

The SafD-dsSafA was successfully constructed and over-expressed in *E. coli* (BL21-AI cells). Test expression experiments allowed selecting the optimal expression conditions, which helped to produce large amounts of protein and purify it to very high homogeneity using cation and anion exchange chromatography and gel filtration.

In order to investigate the polymerization capability of SafD, we performed mass spectrometry analysis and found that SafD does not have any N-terminal extension (Nte) sequence of its own which is essential for polymerization. On the other hand, our melting and chemical denaturation study revealed that SafD-dsSafA is a stable protein, strongly suggesting that in the fiber SafD is complemented with the donor strand of SafA. From this facts, we could concluded that SafD sits at the tip of the native fibre as a single subunit which is donor strand (Nte) complemented by SafA.

To determine the structure of a protein, crystallization is a must and we got some very nice crystals from this protein. One crystal (2 Å resolution) was diffracted in our Lab and the diffracted image is quite promising to solve the structure. Determination of the structure is in progress.

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